Molecular Identification of the Biowarfare Simulant *Serratia marcescens* from a 50-Year-Old Munition Buried at Fort Detrick, Maryland

Guarantor: Chris A. Whitehouse, PhD
Contributors: Chris A. Whitehouse, PhD; Carson Baldwin, MS; Leonard Wasieloski, PhD; John Kondig, BS; LTC John Scherer, MSC USA

serratia marcescens are Gram-negative bacteria that were often used by the U.S. military and others to track movement of bacteria in the environment. As part of ongoing construction at Fort Detrick, Maryland, what appeared to be a small bomblet was found buried in the ground at the site of an old test grid. A sample of a clear, straw-colored liquid was aseptically removed from the plastic reservoir; the results of routine cultures on standard bacteriological media were negative. DNA was extracted from the sample and found to be 99% identical to S. marcescens. These results demonstrate the ability to identify the contents of a biological munition that had been buried for ~50 years.

Introduction

Serratia marcescens is a Gram-negative, saprophytic bacillus classified as a member of the Enterobacteriaceae family. It was originally considered to be an innocuous nonpathogenic organism and was frequently used as a biological marker because of its characteristic production of a red pigment, prodigiosin, which produced easily recognized red colonies when grown on artificial media. For these reasons, *S. marcescens* (along with *Bacillus globigii*) was commonly used by the U.S. military as a bioweapon (BW) simulant in a variety of open-air, mock-BW attack exercises from the 1940s through the 1960s.[1] Since that time, *S. marcescens* has been identified as an important cause of nosocomial infections of the past 30 years, predominantly in immunocompromised patients, and its use as a biological marker in the environment has ceased.[2]

Today, Fort Detrick, Maryland, is home to the U.S. Army Medical Research Institute of Infectious Diseases and is the center of the military's biodefense research efforts. Before 1969, however, Fort Detrick was the site of the U.S. offensive BW program. As part of that program, open-air tests using *S. marcescens* and *B. globigii* were conducted in "area B," an area established as a proving ground for outdoor testing. Area B was laid out in a circular grid, with a series of seven concentric circles, and was designed to test the flow of materials through the air. In its history, area B was also used as a sanitary landfill, a disposal site, and a working farm.[3] At no time was area B ever used to test the release of actual BW agents.

Diagnostic Systems Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD 21702.

The views, opinions, and findings contained herein are those of the authors and should not be construed as an official Department of the Army position, policy, or decision unless so designated by other documentation.

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Case Report

As part of ongoing construction at Fort Detrick, multiple pieces of a biological munition were uncovered at the former location of the area B test grid. These pieces included castings, an impinger and aerosol disperser, and a plastic biological reservoir, which contained ~30 mL of a clear, straw-colored liquid (Fig. 1). The volume and appearance of the liquid were consistent with what would be expected for this type of vessel (W. Patrick, unpublished data). The reservoir was lying on top of the soil, intact, with no visible signs of leakage. By using a pair of sterile surgical gloves, the reservoir was placed in a sealed bag, which was placed inside a 3-inch-diameter, stainless steel container with a lid; the container was sealed and then safely transported to the Diagnostic Systems Division, U.S. Army Medical Research Institute of Infectious Diseases, for analysis.

Laboratory Testing

When the reservoir was received in the laboratory, it was placed in a biological safety cabinet and removed from the secondary packaging for inspection. The reservoir was initially covered with soil residue but appeared otherwise intact. The soil was wiped off with a 10% bleach solution, which revealed that the thick, opaque, plastic reservoir was still sealed, with no visible cracks. An aliquot of the liquid bomb fill was removed aseptically by using a needle and syringe and was cultured both aerobically and anaerobically on routine bacteriological media. Total DNA was extracted from a 200-μL aliquot of the sample by using a QIAamp DNA mini kit (Qiagen, Valencia, California), according to the manufacturer's instructions. All oligonucleo-

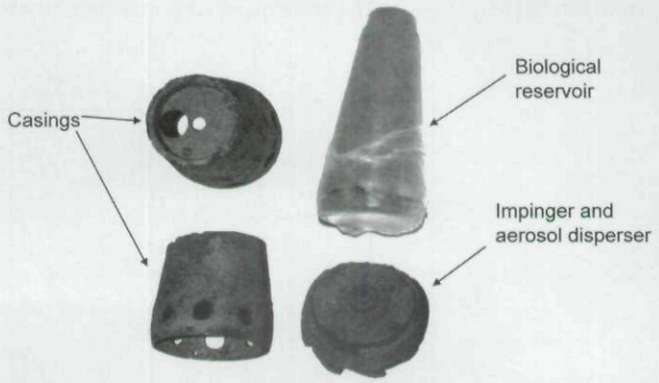

Fig. 1. Recovered pieces of the biological munition found buried in the ground at Fort Detrick. An aliquot of liquid bomb fill was sterilely removed from the biological reservoir for DNA extraction.

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14. ABSTRACT

Serratia marcescens are gram-negative bacteria that were often used by the U.S. military and others to track movement of bacteria in the environment. As part of ongoing construction at Fort Detrick, what appeared to be a small bomblet was found buried in the ground at the site of an old test grid. A sample of clear, straw-colored liquid was aseptically removed from the plastic reservoir and routine culture on standard bacteriological media was negative. DNA was extracted from the sample and found to be 99% identical to S. marcescens. These results demonstrate the ability to identify the contents of a biological munition that had been buried for approximately 50 years.

15. SUBJECT TERMS

Serratia marcescens, old munition, buried munition, identification, DNA extraction

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tide primers used in this study are summarized in Table I. Primers were chosen to amplify the majority of the bacterial 16S rRNA gene[4] or a portion of the RNA polymerase β-subunit-encoding gene *rpoB*.[5] All primers were obtained from Invitrogen Life Technologies (Carlsbad, California). Polymerase chain reaction (PCR) was carried out with a PTC-100 thermal cycler (MJ Research, Ramsey, Minnesota). For the 16S rRNA PCR, reactions were assembled in 50-μL volumes and consisted of 1× PCR buffer, 0.8 mM dNTPs, 1.5 mM $MgCl_2$, 5 U of AmpliTaq Gold (Applied Biosystems, Foster City, California), and 250 nM levels of each primer. PCR amplification was performed by using the following cycling conditions: 10 minutes at 95°C, followed by eight cycles of 95°C for 30 seconds, 48°C for 30 seconds, and 72°C for 30 seconds and then 37 cycles of 95°C for 15 seconds, 56°C for 20 seconds, and 72°C for 20 seconds. For the *rpoB* PCR, reactions were carried out in a 40-μL volume containing 1× PCR buffer, 0.32 mM dNTPs, 1.5 mM $MgCl_2$, 3 U of FastStart Taq (Roche Applied Science, Indianapolis, Indiana), and 250 nM levels of each primer. PCR amplification was performed under the following cycling conditions: 10 minutes at 95°C, followed by eight cycles of 95°C for 30 seconds, 48°C (increasing 0.9°C per cycle) for 30 seconds, and 72°C for 30 seconds and then 37 cycles of 95°C for 15 seconds, 56°C for 20 seconds, and 72°C for 20 seconds. Electrophoresis was performed on 4% NuSieve 3:1 agarose gels (Cambrex, E. Rutherford, New Jersey) by using 1× Tris/borate/ethylenediaminetetraacetic acid buffer at 120 V for 30 minutes. Gels were imaged with an AlphaImager (Alpha Innotech, San Leandro, California) to determine whether the appropriate amplicon was present for sequencing. PCR amplicons were purified by using a QIAquick PCR purification kit (Qiagen, Chatsworth, California), according to the manufacturer's instructions. Approximately 10 ng of purified DNA was used as the template in BigDye Terminator v1.1 Cycle Ready Reactions (Applied Biosystems) with the primers in Table I; sequencing was performed with an ABI Prism 3100 genetic analyzer (Applied Biosystems) according to the manufacturer's instructions. For the 16S rRNA gene, sequences were assembled into a single contiguous sequence consisting of 1,469 nucleotides with DNAStar SeqManII software (DNAStar, Madison, Wisconsin). Sequences were compared with those available in GenBank by using the Basic Local Alignment Search Tool.[6] Phylogenetic relationships were inferred by comparing partial sequences of the 16S rRNA and *rpoB* genes. Sequences were aligned by using the multisequence alignment program GeneDoc (version 2.6.02)

TABLE I

PCR PRIMER SEQUENCES USED IN THIS STUDY

Primer Name	Primer Sequence (5′ to 3′)	Position[a]
16S rRNA		
27f	AGAGTTTGATYMTGGCTCAG	8–27
355f	ACTCCTACGGGAGGCAGC	338–355
338r	GCTGCCTCCCGTAGGAGT	338–355
556r	CTTTACGCCCARTRAWTCCG	556–575
787r	GGACTACCAGGGTATCTAAT	787–806
806f	ATTAGATACCCTGGTAGTCC	787–806
926r	AAACTYAAAKGAATTGACGG	907–926
930f	TCAAAKGAATTGACGGGGGC	911–930
1175r	ACGTCATCCCCACCTTCCTC	1,175–1,194
1371r	AGGCCCGGGAACGTATTCAC	1,390–1,371
1525r	AAGGAGGTGWTCCARCC	1,525–1,541
rpoB		
359f	TTATCGCTCAGGCGAACTCCAAC	1,286–1,308
359r	TGCTGGATTCGCCTTTGCTACG	1,185–1,206

[a] Location in *Escherichia coli* genes.

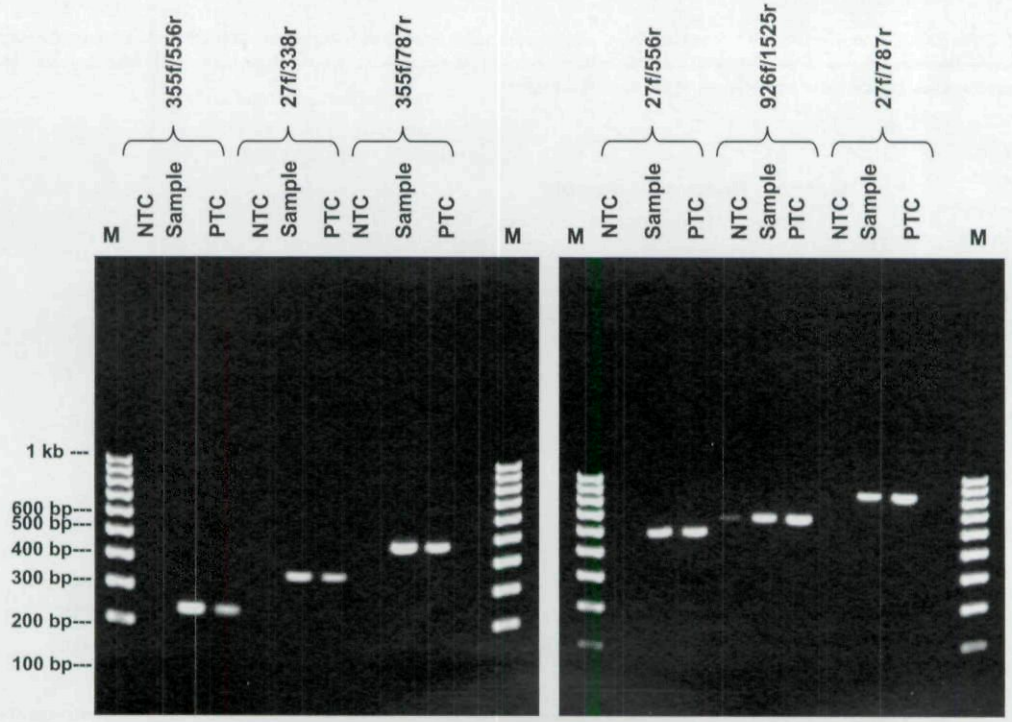

Fig. 2. Agarose gel electrophoresis of 16S rRNA PCR products amplified from the liquid bomb fill. M, molecular weight marker; NTC, no-template control (water); PTC, positive template control (*Escherichia coli*).

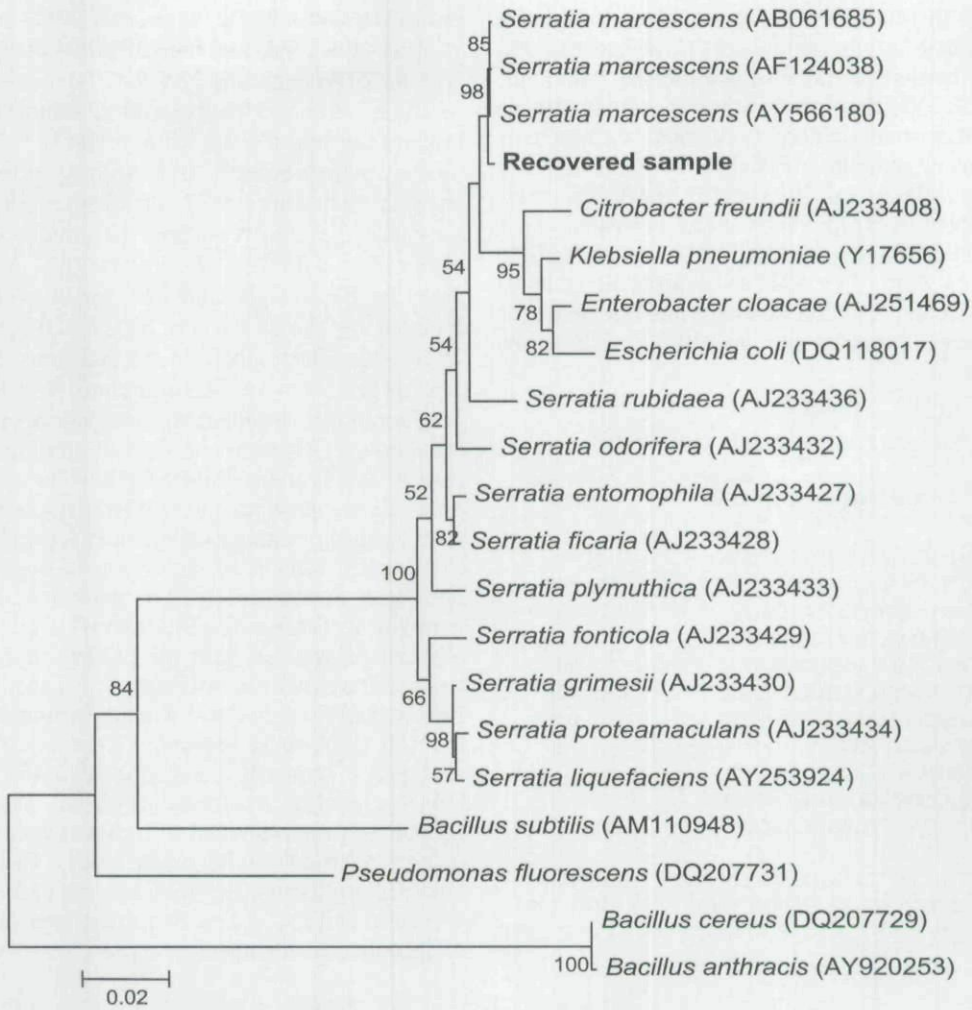

Fig. 3. Phylogenetic tree derived from the 16S rRNA gene sequences. The lengths of the horizontal branches are proportional to the number of nucleotide differences between bacterial species. Bootstrap values above 50%, obtained from 500 replicates of the analysis, are shown at the appropriate branch points. The GenBank accession numbers of the bacterial species are shown in parentheses. Scale bar, 2% divergence.

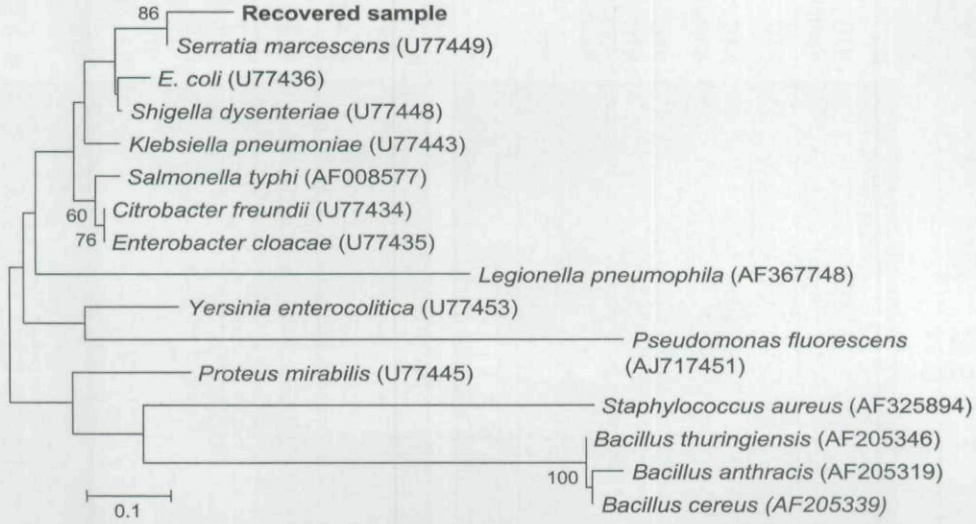

Fig. 4. Phylogenetic tree derived from the partial *rpoB* gene sequences. The lengths of the horizontal branches are proportional to the number of nucleotide differences between bacterial species. Bootstrap values above 50%, obtained from 500 replicates of the analysis, are shown at the appropriate branch points. The GenBank accession numbers of the bacterial species are shown in parentheses. Scale bar, 10% divergence.

and were analyzed with a neighbor-joining method with Kimura two-parameter distances by using MEGA software (version 2.1). The 16S and *rpoB* sequences for the recovered *S. marcescens* sample were deposited in the GenBank database under accession numbers DQ501957 and DQ520586, respectively.

Attempts at culturing the liquid bomb fill both aerobically and anaerobically, on routine microbiological media, yielded negative results. DNA was extracted from an aliquot of the liquid and subjected to 16S rRNA PCR. Multiple overlapping amplicons that covered the majority of the bacterial 16S rRNA gene were generated (Fig. 2). The sequences of the individual 16S rRNA amplicons were assembled into a 1,469-base pair contiguous sequence and submitted to a Basic Local Alignment Search Tool database search, which showed 99.86% similarity to two *S. marcescens* entries. The closest matches were to *S. marcescens* subsp. *sakuensis* (GenBank accession no. AB0616185), a unique, spore-forming, *S. marcescens* strain originally isolated from a wastewater treatment tank in Japan,[7] and *S. marcescens* (GenBank accession no. AB061685), which was part of the bacterial flora of the sheep scab mite.[8] The presence of *S. marcescens* in the sample was confirmed by performing PCR and sequencing an additional gene target (*rpoB*). The *rpoB* gene encodes the bacterial RNA polymerase β-subunit and has been extensively used for sequence-based identification and molecular phylogeny of bacterial pathogens.[9–13] PCR using primers targeting conserved regions of the *rpoB* gene yielded an amplicon of the expected size (data not shown). Sequence analysis of the amplicon revealed 100% (42 of 42 positions) nucleotide sequence identity to *S. marcescens* (GenBank accession no. U77449). Phylogenetic analysis showed the relationships of the sequence recovered from the liquid bomb fill to other bacterial 16S rRNA (Fig. 3) and *rpoB* (Fig. 4) sequences.

Discussion

This study represents the first comprehensive examination of the contents of a biological munition recovered from the time of the U.S. offensive BW program (1942–1969). Although the exact age of the munition is not known, the munition was estimated to be between 46 and 52 years old, based on when this type of munition was used at Fort Detrick (W. Patrick, personal communication).

Multiple precautions were taken to avoid cross-contamination of samples, including conducting sample processing, PCR, and post-PCR analysis in separate rooms. Other precautions were taken from recently published criteria for the authentication of molecular data in paleomicrobiology[14] and included (i) not using a positive control, (ii) using a new primer sequence that had not previously been amplified in the laboratory, (iii) sequencing the amplicon to confirm its identity, and (iv) confirming a positive result by amplifying and sequencing a second target.

S. marcescens, which was once thought to be an innocuous soil bacterium, was often used as a biological marker during the U.S. offensive BW program. In fact, this is not the first time a BW simulant has been identified from the liquid bomb fill of a buried biological munition. In 1995, construction workers at Wright-Patterson Air Force Base, near Dayton, Ohio, uncovered a buried cache of biological munitions, which were shown to contain nonviable *Brucella suis*.[15] To our knowledge, however, this is the first report of *S. marcescens* being identified from a biological munition buried in the ground for ~50 years. Our results are consistent with *S. marcescens* being used as a BW simulant and highlight the use of molecular techniques to detect and to identify bacterial organisms from the past that can no longer be cultivated.

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